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PA NT COOPERATION TREAT

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

SKELTON, Stephen, Richard
D/IPR
Formalities Section
Poplar 2, MOD Abbey Wood #19
Bristol BS34 8JH
ROYAUME-UNI

Date of mailing (day/month/year)
14 June 2001 (14.06.01)

Applicant's or agent's file reference
P1246/WOD

IMPORTANT NOTIFICATION

International application No.
PCT/GB00/03402

International filing date (day/month/year)
06 September 2000 (06.09.00)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

Name and Address
BOWDERY, A., O.
D/IPR
Formalities Section
Poplar 2, MOD Abbey Wood #19
Bristol BS34 8JH
United Kingdom

State of Nationality
State of Residence
Telephone No.
0117 91 32857
Facsimile No.
0117 91 32930
Teleprinter No.

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [ ] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address
SKELTON, Stephen, Richard
D/IPR
Formalities Section
Poplar 2, MOD Abbey Wood #19
Bristol BS34 8JH
United Kingdom

State of Nationality
State of Residence
Telephone No.
0117 91 32857
Facsimile No.
0117 91 32930
Teleprinter No.

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[X] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
Anman QIU

Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994)

004089539

PA NT COOPERATION TREAT

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

SKELTON, Stephen, Richard
D/IPR
Formalities Section
Poplar 2, MOD Abbey Wood #19
Bristol BS34 8JH
ROYAUME-UNI

Date of mailing (day/month/year)
14 June 2001 (14.06.01)

Applicant's or agent's file reference
P1246/WOD

IMPORTANT NOTIFICATION

International application No.
PCT/GB00/03402

International filing date (day/month/year)
06 September 2000 (06.09.00)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address
THE SECRETARY OF STATE FOR DEFENCE
Defence Evaluation and Research Agency
Ively Road
Farnborough
Hampshire GU14 0LX
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
THE SECRETARY OF STATE FOR DEFENCE
CBD Porton Down
Salisburyd
Wiltshire SP4 0JQ
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
Anman QIU
Telephone No.: (41-22) 338.83.38

PA NT COOPERATION TREAT

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 14 June 2001 (14.06.01)	
International application No. PCT/GB00/03402	**Applicant's or agent's file reference** P1246/WOD
International filing date (day/month/year) 06 September 2000 (06.09.00)	**Priority date** (day/month/year) 10 September 1999 (10.09.99)
Applicant TITBALL, Richard, William et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

14 March 2001 (14.03.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Anman QIU
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PA NT COOPERATION TREAT

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

SKELTON, Stephen, Richard
D/IPR
Formalities Section
Poplar 2, MOD Abbey Wood #19
Bristol BS34 8JH
ROYAUME-UNI

Date of mailing (day/month/year)
24 July 2001 (24.07.01)

Applicant's or agent's file reference
P1246/WOD

IMPORTANT NOTIFICATION

International application No.
PCT/GB00/03402

International filing date (day/month/year)
06 September 2000 (06.09.00)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

Name and Address

BOWDERY, A., O.
D/IPR
Formalities Section
Poplar 2, MOD Abbey Wood #19
Bristol BS34 8JH
United Kingdom

State of Nationality

State of Residence

Telephone No.
0117 91 32857

Facsimile No.
0117 91 32930

Teleprinter No.

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [ ] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address

SKELTON, Stephen, Richard
D/IPR
Formalities Section
Poplar 2, MOD Abbey Wood #19
Bristol BS34 8JH
United Kingdom

State of Nationality

State of Residence

Telephone No.
0117 91 32857

Facsimile No.
0117 91 32930

Teleprinter No.

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Anman QIU

Telephone No.: (41-22) 338.83.38

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PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

SKELTON, Stephen, Richard
D/IPR
Formalities Section
Poplar 2, MOD Abbey Wood #19
Bristol BS34 8JH
ROYAUME-UNI

Date of mailing (day/month/year)
25 July 2001 (25.07.01)

Applicant's or agent's file reference
P1246/WOD

IMPORTANT NOTIFICATION

International application No.
PCT/GB00/03402

International filing date (day/month/year)
06 September 2000 (06.09.00)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address
THE SECRETARY OF STATE FOR DEFENCE
CBD Porton Down
Salisburyd
Wiltshire SP4 0JQ
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
THE SECRETARY OF STATE FOR DEFENCE
DSTL
Porton Down
Salisbury
Wiltshire SP4 0JQ
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
Anman QIU

Telephone No.: (41-22) 338.83.38

PA NT COOPERATION TREA

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

SKELTON, Stephen, Richard
D/IPR
Formalities Section
Poplar 2, MOD Abbey Wood #19
Bristol BS34 8JH
ROYAUME-UNI

Date of mailing (day/month/year)
13 November 2001 (13.11.01)

Applicant's or agent's file reference
P1246/WOD

IMPORTANT NOTIFICATION

International application No.
PCT/GB00/03402

International filing date (day/month/year)
06 September 2000 (06.09.00)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address
TITBALL, Richard, William
CBD Porton Down
Salisbury
Wiltshire SP4 0JQ
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
TITBALL, Richard, William
DSTL
Porton Down
Salisbury
Wiltshire SP4 0JQ
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
Anman QIU
Telephone No.: (41-22) 338.83.38

PA. ENT COOPERATION TREA.

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

SKELTON, Stephen, Richard
D/IPR
Formalities Section
Poplar 2, MOD Abbey Wood #19
Bristol BS34 8JH
ROYAUME-UNI

Date of mailing (day/month/year)
05 février 2002 (05.02.02)

Applicant's or agent's file reference
P1246/WOD

IMPORTANT NOTIFICATION

International application No.
PCT/GB00/03402

International filing date (day/month/year)
06 septembre 2000 (06.09.00)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address
BULLIFENT, Helen, Lisa
CBD Porton Down
Salisbury
Wiltshire SP4 0JQ
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
BULLIFENT, Helen, Lisa
Dstl
Porton Down
Salisbury
Wiltshire SP4 0JQ
United Kingdom

State of Nationality: GB
State of Residence: GB
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
Anman QIU

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

CBO AMI
IPR1 RECEIVED
10 DEC 2001
MOD-DPA

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

SKELTON, Srephen Richard
D/IPR Formalities Ssction
Poplar 2
MOD Abbey Wood 19
Bristol BS34 8JH
GRANDE BRETAGNE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	06.12.2001
Applicant's or agent's file reference: P1246/WOD	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/03402	06/09/2000	10/09/1999

Applicant
THE SECRETARY OF STATE FOR DEFENCE et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Cleere, C Tel.+49 89 2399-7713

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P1246/WOD		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/03402	06/09/2000	10/09/1999
International Patent Classification (IPC) or national classification and IPC C12N15/67		
Applicant THE SECRETARY OF STATE FOR DEFENCE et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
14/03/2001	06.12.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Nichogiannopoulou, A Telephone No. +49 89 2399 8054

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/03402

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-21 as originally filed

Claims, No.:

1-16 as originally filed

Drawings, sheets:

1/9-9/9 as originally filed

Sequence listing part of the description, pages:

1-5, filed with the letter of 09.03.2001

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

   Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
   see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 1-16, all partially.

because:

- ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for the said claims Nos. 1-16, all partially.

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-16
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-16
Industrial applicability (IA)	Yes:	Claims	1-14, 16
	No:	Claims	

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

Re Item II

Priority

1. The present application validly claims priority from 10.09.1999. Any documents cited in the International Search Report as P documents have therefore not been considered as comprised in the prior art relevant for the present application.

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. No meaningful examination could be performed for claims 1-16, all partially, for the following reasons:

1.1. Rule 66. 1.(e) (PCT):

No complete international search report has been established for said claims (see Form PCT/ISA/210 issued on 12/06/2001). Accordingly, said claims need not be the subject of international preliminary examination.

2. Claim 15 -as far as it concerns *in vivo* methods- relates to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of this claim (Article 34(4)(a)(i) PCT).

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:

   D1: TITBALL R W ET AL: 'Expression of the Yersinia pestis capsular antigen (F1 antigen) on the surface of an aroA mutant of Salmonella typhimurium induces high levels of protection against plague.' INFECTION AND IMMUNITY, vol. 65, no. 5, 1997, pages 1926-1930, XP002164415 ISSN: 0019-9567

   D2: WO 96 28551 A (BENNETT A M; LEARY S E C (GB); TITBALL R) 19 September 1996 (1996-09-19)

   D3: HOHMANN E L ET AL: 'Macrophage-inducible expression of a model antigen in Salmonella typhimurium enhances immunogenicity.' PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 92, no. 7, 1995, pages 2904-2908, XP002164416 1995 ISSN: 0027-8424

2. **Novelty** (Article 33(2) PCT)

   The present application discloses the transformation of gut-colonising microorganisms (e.g. *Salmonella spp.*) with an immunogenic protein (e.g. F1 antigen of *Yersinia pestis*) under the control of the phoP promoter, leading to enhanced expression of the immunogenic protein at mucosal effector sites.

   Expression of an immunogenic protein at a mucosal effector site under the control of the phoP promoter has not been disclosed in the available prior art. The subject-matter of claims 1-16 appears thus to be novel under the terms of Article 33(2) PCT.

3. **Inventiv st p** (Article 33(3) PCT)

3.1. **D1** is a publication by one of the inventors of the present application disclosing the

immunisation of mice with an attenuated *Salmonella* strain expressing the F1 antigen of *Yersinia pestis* under the control of the *Yersinia* caf operon.
D2 discloses *Salmonella* expressing *Yersinia pestis* F1 antigen as a vehicle for vaccination against *Y. pestis*. The antigens are expressed under the control of a lac promoter but the use of other promoters such as the macrophage promoter (nirB) is specifically contemplated (page 5, lines 14-15).
D3 discloses the expression of heterologous antigens in *Salmonella* under the control of the induced pagC locus, which is activated within macrophages.
Given the current need for recombinant vaccines the skilled person would have combined the teachings of **D1**, **D2** and **D3** to arrive at the subject-matter of the present application without undue burden. The subject-matter of claims 1-16 is thus considered to lack an inventive step under the terms of Article 33(3) PCT.

4. **Industrial applicability** (Article 33(4) PCT)
The subject-matter of claims on which an opinion has been formed (see item III) appears to be industrially applicable under the terms of Article 33(4) PCT.

Re Item VII

Certain defects in the international application

1. Contrary to the requirements of Rule 5.1(ii) PCT, documents **D1-D3** are not identified in the description and the relevant background art disclosed therein is not briefly discussed.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
P1246/WOD		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 00/ 03402	06/09/2000	10/09/1999
Applicant		
THE SECRETARY OF STATE FOR DEFENCE, DEFENCE EVA...		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [X] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [X] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 00/03402

Box I Observations wher certain claims w re f und unsearchabl (Continuati n f item 1 f first sh et)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 15 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-16 all partially

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-16 all partially

A method for enhancing expression of a desired protein at mucosal effector sites, comprising placing said protein under the control of the promoter of the phoP gene (SEQ ID No:2), a virulence gene induced in the phagosomal compartment of host cells. Constructs comprising said promoter, recombinant gut-colonising microorganisms transformed with said constructs, vaccines comprising said recombinant microorganisms and a method of inducing an immune response with said recombinant microorganisms.

2. Claims: 1-16 all partially

A method for enhancing expression of a desired protein at mucosal effector sites, comprising placing said protein under the control of the promoter of the pagC gene (SEQ ID No:3), a gene which encodes an envelope protein required for survival in the macrophage. Constructs comprising said promoter, recombinant gut-colonising microorganisms transformed with said constructs, vaccines comprising said recombinant microorganisms and a method of inducing an immune response with said recombinant microorganisms.

3. Claims: 1-16 all partially

A method for enhancing expression of a desired protein at mucosal effector sites, comprising placing said protein under the control of the promoter of the ompC gene (SEQ ID No:4), a gene upregulated under conditions of high osmotic strength, such as those found within the gut. Constructs comprising said promoter, recombinant gut-colonising microorganisms transformed with said constructs, vaccines comprising said recombinant microorganisms and a method of inducing an immune response with said recombinant microorganisms.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/03402

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/67 C12N1/21 A61K39/02 C12N1/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, PAJ, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TITBALL RICHARD W ET AL: "Expression of the Yersinia pestis capsular antigen (F1 antigen) on the surface of an aroA mutant of Salmonella typhimurium induces high levels of protection against plague." INFECTION AND IMMUNITY, vol. 65, no. 5, 1997, pages 1926-1930, XP002164415 ISSN: 0019-9567 the whole document --- -/--	1-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 March 2001	12.06.01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nichogiannopoulou, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HOHMANN ELIZABETH L ET AL: "Macrophage-inducible expression of a model antigen in Salmonella typhimurium enhances immunogenicity." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 92, no. 7, 1995, pages 2904-2908, XP002164416 1995 ISSN: 0027-8424 the whole document ---	1-16
Y	WO 96 28551 A (BENNETT ALICE MARIE ;LEARY SOPHIE EMMA CLARE (GB); TITBALL RICHARD) 19 September 1996 (1996-09-19) page 5, line 14 - line 15 ---	1-16
A	ROBERTS MARK ET AL: "Oral vaccination against tetanus: Comparison of the immunogenetics of Salmonella strains expressing fragment C from the nirB and htrA promoters." INFECTION AND IMMUNITY, vol. 66, no. 7, July 1998 (1998-07), pages 3080-3087, XP002164417 ISSN: 0019-9567 cited in the application the whole document ---	1-16
A	MCSORLEY STEPHEN J ET AL: "Vaccine efficacy of Salmonella strains expressing glycoprotein 63 with different promoters." INFECTION AND IMMUNITY, vol. 65, no. 1, 1997, pages 171-178, XP002164418 ISSN: 0019-9567 cited in the application the whole document ---	1-16
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2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/03402

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9628551 A	19-09-1996	AU 710181 B	16-09-1999
		AU 4951196 A	02-10-1996
		CA 2215203 A	19-09-1996
		CN 1184505 A	10-06-1998
		EP 0815235 A	07-01-1998
		JP 11501654 T	09-02-1999
		US 5985285 A	16-11-1999
		ZA 9602036 A	16-07-1996

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 March 2001 (22.03.2001)

PCT

(10) International Publication Number
WO 01/19974 A3

(51) International Patent Classification[7]: C12N 15/67, 1/21, A61K 39/02, C12N 1/20

(21) International Application Number: PCT/GB00/03402

(22) International Filing Date:
6 September 2000 (06.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9921275.5 10 September 1999 (10.09.1999) GB
0017000.1 12 July 2000 (12.07.2000) GB

(71) Applicant *(for all designated States except US)*: **THE SECRETARY OF STATE FOR DEFENCE** [GB/GB]; DSTL, Porton Down, Salisbury, Wiltshire SP4 0JQ (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **TITBALL, Richard, William** [GB/GB]; CBD Porton Down, Salisbury, Wiltshire SP4 0JQ (GB). **BULLIFENT, Helen, Lisa** [GB/GB]; CBD Porton Down, Salisbury, Wiltshire SP4 0JQ (GB).

(74) Agent: **SKELTON, Stephen, Richard**; D/IPR, Formalities Section, Poplar 2, MOD Abbey Wood #19, Bristol BS34 8JH (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
15 November 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/19974 A3

(54) Title: RECOMBINANT MICROORGANISMS

(57) Abstract: A method of enhancing expression of a desired protein at mucosal effector sites, said method comprising placing the protein to be expressed under the control of a promoter having SEQ ID NO 2, SEQ ID NO 3 or SEQ ID NO 4 or a fragment or variant r any of these which has promoter activity, and causing expression in mucosal cells. Constructs used in the methods, as well as suitable recombinant gut-colonising microorganisms such as a *Salmonella spp.* are also described and claimed. Such organisms are useful in the preparation of vaccines.

INTERNATIONAL SEARCH REPORT

Inte al Application No
PCT/GB 00/03402

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/67 C12N1/21 A61K39/02 C12N1/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, PAJ, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of box C.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 March 2001	12.06.01

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nichogiannopoulou, A

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INTERNATIONAL SEARCH REPORT

Inter ..onal Application No
PCT/GB 00/03402

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HOHMANN ELIZABETH L ET AL: "Macrophage-inducible expression of a model antigen in Salmonella typhimurium enhances immunogenicity." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 92, no. 7, 1995, pages 2904-2908, XP002164416 1995 ISSN: 0027-8424 the whole document ---	1-16
Y	WO 96 28551 A (BENNETT ALICE MARIE ;LEARY SOPHIE EMMA CLARE (GB); TITBALL RICHARD) 19 September 1996 (1996-09-19) page 5, line 14 - line 15 ---	1-16
A	ROBERTS MARK ET AL: "Oral vaccination against tetanus: Comparison of the immunogenetics of Salmonella strains expressing fragment C from the nirB and htrA promoters." INFECTION AND IMMUNITY, vol. 66, no. 7, July 1998 (1998-07), pages 3080-3087, XP002164417 ISSN: 0019-9567 cited in the application the whole document ---	1-16
A	MCSORLEY STEPHEN J ET AL: "Vaccine efficacy of Salmonella strains expressing glycoprotein 63 with different promoters." INFECTION AND IMMUNITY, vol. 65, no. 1, 1997, pages 171-178, XP002164418 ISSN: 0019-9567 cited in the application the whole document ---	1-16
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2

INTERNATIONAL SEARCH REPORT

I.. .national application No.
PCT/GB 00/03402

Box I Observations where c rtain claims were found unsearchable (Continuation f item 1 f first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 15 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

B x II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-16 all partially

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-16 all partially

A method for enhancing expression of a desired protein at mucosal effector sites, comprising placing said protein under the control of the promoter of the phoP gene (SEQ ID No:2), a virulence gene induced in the phagosomal compartment of host cells. Constructs comprising said promoter, recombinant gut-colonising microorganisms transformed with said constructs, vaccines comprising said recombinant microorganisms and a method of inducing an immune response with said recombinant microorganisms.

2. Claims: 1-16 all partially

A method for enhancing expression of a desired protein at mucosal effector sites, comprising placing said protein under the control of the promoter of the pagC gene (SEQ ID No:3), a gene which encodes an envelope protein required for survival in the macrophage. Constructs comprising said promoter, recombinant gut-colonising microorganisms transformed with said constructs, vaccines comprising said recombinant microorganisms and a method of inducing an immune response with said recombinant microorganisms.

3. Claims: 1-16 all partially

A method for enhancing expression of a desired protein at mucosal effector sites, comprising placing said protein under the control of the promoter of the ompC gene (SEQ ID No:4), a gene upregulated under conditions of high osmotic strength, such as those found within the gut. Constructs comprising said promoter, recombinant gut-colonising microorganisms transformed with said constructs, vaccines comprising said recombinant microorganisms and a method of inducing an immune response with said recombinant microorganisms.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ɔnal Application No
PCT/GB 00/03402

Patent document cited in search report	Publication date	Patent family member(s)	Publication dat
WO 9628551 A	19-09-1996	AU 710181 B	16-09-1999
		AU 4951196 A	02-10-1996
		CA 2215203 A	19-09-1996
		CN 1184505 A	10-06-1998
		EP 0815235 A	07-01-1998
		JP 11501654 T	09-02-1999
		US 5985285 A	16-11-1999
		ZA 9602036 A	16-07-1996

Recombinant microorganisms

The present invention relates to recombinant microorganisms, in particular gut-colonising organisms, which are useful for example in the delivery of antigenic material and thus form the basis of vaccines. Vaccines comprising these organisms and promoter sequences for use in them form a further aspect of the invention.

Attenuated mutants of *Salmonella typhi* (e.g. *aroA*, *aroC*, *htrA*) are currently being evaluated as live, oral vaccines against typhoid fever (Tacket CO, et al., Infect. Immun. 1997;65:452-6). These mutants have also attracted attention as carriers for guest (vaccine) antigens but suitable animal models for testing these vaccines are not available. In view of this, many workers have used *Salmonella typhimurium aroA* expressing guest antigens for investigating the immune responses induced after oral vaccination of mice.

The unregulated expression of foreign genes within *Salmonella* species such as *S. typhimurium* can lead to plasmid instability, yet the stable expression of the guest antigen at the appropriate site in the body is necessary for the induction of a protective response. One approach to promote the stable expression of guest antigens involves the chromosomal integration of the heterologous gene. However, this may reduce the immune response because of gene dosage effects (Covone M G, et al., Infect. Immun. 1998;66:224-31).

The balanced lethal system (Curtiss R III, et al., Res. Microbiol. 1990;141:797-805, Nakayama K, et al., Bio/Technology 1988;6:693-97) relies on the complementation of a lethal mutation by a plasmid which also encodes the guest antigen. Whilst this ensures retention of the plasmid, the gene encoding the guest antigen itself may be deleted. An alternative approach involves the use of promoters which are induced within

host tissues to direct guest antigen expression at that site. Because the gene is only expressed after certain environmental cues have been recognised, this approach might reduce the selective pressure towards deleting the gene.

This solution to the problem of expression of guest antigens has also been identified by other workers. A variety of antigens have been expressed in *S. typhimurium* from the *nirB* promoter which is upregulated under anaerobic conditions and within host cells (Oxer M D, et al. Nucleic Acids Res. 1991;19:2889-92). Guest antigens delivered using the *nirB* promoter system induce superior responses than the same antigens delivered from a constitutive promoter. In addition, the *nirB* promoter-driven genes were maintained more effectively in the *Salmonella* host strain. More recently, it has been shown that the *htrA* and *osmC* promoter can be used to direct expression of guest antigens in *Salmonella* (McSorley S J, et al., Infect. Immun. 1997;65:171-78, Roberts M, et al., Infect. Immun. 1998;66:3080-87). However, it is likely that these promoters will not be suited to the expression of all guest antigens.

Immunisation with the F1-antigen of *Y. pestis* has previously been shown to induce an antibody-mediated protective response against plague (Green M, et al., FEMS Microbiology and Immunology, 1998;23:107-13) and we have previously shown that the F1-antigen can be expressed in *S. typhimurium* (Oyston P C F, et al., Infect. Immun. 1995;63:563-68, Titball R W, et al., Infect. Immun. 1997;65:1926-30). The antigenic properties of F1-antigen have been exploited to investigate the ways in which different promoters, which are induced at different sites in the body, can be used to induce different antibody responses to guest antigens expressed in *S. typhimurium*. It is known that the invasion and spread of *S. typhimurium* within the host is accompanied by the expression of different subsets of genes which are involved in processes such as attachment and invasion,

penetration of the epithelium and the infection of deep lymphoid tissue.

The OmpR/EnvZ two component regulatory system responds to changes in the osmotic strength and pH within *S. typhimurium* (Foster J W, et al., Microbiology 1994;140:341-52). It has been suggested that this system might play a role in allowing the bacterium to survive in the gut by regulating the expression of outer membrane porins such as OmpC (Pratt L A, et al., American Society for Microbiology, ASM Press, Washington DC, 1995, pp105-27, Nikaido H, et al., Cellular and Molecular Biology. American Society for Microbiology, Washington DC. 1987, pp7-22, García Véscovi E. et al., Cell. 1996;84:165-74).

The PhoP/PhoQ two-component regulatory system controls virulence properties such as survival within macrophages, resistance to host defence antimicrobial peptides and acid pH, invasion of epithelial cells, the formation of spacious vacuoles and the processing and presentation of antigens by activated macrophages (Miller S I. et al., Proc. Natl. Acad, Sci USA 1989;86:5054-58, Fields P I, et al., Science 1989;243:1059-62, Pegues D A, et al., Mol. Microbiol. 1995;17:169-81, Wick M J, et al., Mol. Microbiol. 1995;16:465-76), in response to environmental magnesium concentration (García Véscovi E. et al., Cell. 1996;84:165-74). Over forty genes are regulated by this system in *S. typhimurium* (Soncini F C, et al., J. Bacteriol. 1996;178:5092-99) including the *phoP* gene, which is autoregulated (Soncini F C, et al., J. Bacteriol. 1995;177:4364-71) and the *pagC* gene which encodes an envelope protein required for survival in the macrophage (Alpuche-Aranda C M, et al., Proc. Natl. Acad. Sci. USA 1992;89:10079-83).

Attenuation of Salmonella by partial deletion of the pagC gene and fusion to a heterologous protein is described in USP 5,733,760.

The applicants have however found that certain promoters can be used advantageously in such systems to drive high levels of expression of heterologous proteins, in particular in mucosal cells.

Thus, the present invention provides a method of enhancing expression of a desired protein at mucosal effector sites, said method comprising placing the protein to be expressed under the control of a promoter having SEQ ID NO 2, SEQ ID NO 3 or SEQ ID NO 4 or a fragment or variant or any of these which has promoter activity, and causing expression in mucosal cells.

Further according to the present invention, there is provided a construct comprising a promoter selected from the P_{ompC}, P_{phoP} and P_{pagC} or fragments or variants thereof which can act as promoters, operatively interconnected with a nucleic acid which encodes a protein, able to induce a protective immune response against an organism, in a mammal to which it is administered, wherein said construct contains no further elements of the ompC, phoP or pagC gene.

The present invention further provides a recombinant gut-colonising microorganism which comprises a promoter selected from the P_{ompC}, P_{phoP} and P_{pagC} or fragments or variants thereof which can act as promoters, said promoter being operatively interconnected with a nucleic acid which encodes a heterologous protein, able to induce a protective immune response against a different organism, in a mammal to which it is administered.

In particular, the microorganism has been transformed with the construct described above.

The term "heterologous protein" refers to proteins which are not native to the microorganism strain.

The three promoters (P_{phoP}, P_{pagC} and P_{ompC},) which are included in the organisms of the invention are induced at different stages in the infection process, and hence at different sites in the body. This approach allows the induction of different immune responses which provide protection against pathogens which colonise different host cell compartments. The sequence of these promoters has been elucidated previously, and these are given hereinafter in Figure 6 as SEQ ID NOS 2, 3 and 4 respectively.

Their expression has been compared to that of the constitutively expressed *lacZ* gene promoter. As a result, recombinant gut-colonising microorganisms wherein antigen expression is driven by P_{phoP} promoter forms a preferred embodiment of the invention.

The development of effective vaccines against pathogens is dependent not only on the identification of the appropriate protective antigens but also on the induction of an immune response at the site in the body which provides maximum protection against disease. For some pathogens, serum antibody provides protection against disease. However, many pathogens enter the body at a mucosal surface and protection against these diseases might therefore be dependent on the induction of mucosal immune responses. The *Salmonella* vaccine vector system is ideally suited to the delivery of many vaccine antigens since the vaccine delivery mechanism accurately mimics the natural disease, entering the body via the gut.

Thus in particular embodiment, the recombinant gut-colonising microorganism comprises a *Salmonella spp.* such as *Salmonella typhimurium* or *Salmonella typhi*.

The recombinant *Salmonella* evaluated here showed significant differences in their abilities to induce mucosal IgA antibody responses. Serum IgA levels were not a good predictor of mucosal IgA levels, in accordance with the general findings by

other workers that these responses are not well correlated [Lu FX et al., Infect. Immun. 1999;67:6321-8; Russell MW et al., Infect. Immun. 1991;59:4061-70; and Wenneras C et al., Infect. Immun. 1999;67:6231-41]. After oral dosing all of the recombinant *Salmonella* would have entered the body via M-cells, and, if sufficient antigen was subsequently presented to immune effector cells then mucosal antibody responses would be expected. The finding that mucosal antibody in the gut was induced only after immunisation with recombinant *Salmonella* expressing F1-antigen from the *phoP* or *pagC* gene promoters suggests that these promoters directed high-level expression of F1-antigen within GALT. Peyer's patch cells taken from mice immunised with SL3261 / pP_{pagC}-F1 or SL3261 / pP_{phoP}-F1 produced the highest levels of IgA supporting this suggestion.

Immunisation with *Salmonella* containing pP_{phoP}-F1 also resulted in detectable IgA antibody in the lungs. This is in accordance with the finding that this recombinant also induced the highest levels of IgA in the gut and might indicate that the SL3261 / pP_{phoP}-F1 was more effective than SL3261 / pP_{pagC}-F1 in inducing long-term expression of IgA.

Recombinant gut-colonising microorganisms of the invention are suitably attenuated so that the host does not experience significant harmful effects as a result of infection by the microorganism. Examples of attenuated mutants include *aro* mutants such as *aroA* and *aroC* mutants, apartate β-semi-aldehyde dehydrogenase (ASD) mutants, purine biosynthesis mutants, branched chain amino acid biosynthesis mutants, galactose epimerase (*galE*) mutants, regulatory mutants such as *phoP* and *phoQ* mutants, htrA serine protease mutants and adenyl cyclase mutants. Particular attenuated strains of *Salmonella*, such as *Salmonella typhi* include *aroA, aroC and htrA* mutants or triple mutants including all three mutations.

Recombinant gut-colonising microorganism as described above can be used to deliver a variety of antigenic agents which can be used to induce a protective immune response against a wide range of pathogens. Pathogens which may be targeted in this way are those of humans or animals and include those listed in the Health and Safety Executive: "Categorisation of Biological Agents according to Hazard and Category of Containment", HMSO, ISBN 0717610381. Particular examples of antigenic agents which may be included in the recombinant organisms of the invention include those protective against tetanus such as tetanus toxin H_C fragment, those protective against Botulinum such as *botulinum* toxin H_C fragment, those protective against *Bacillus anthracis* such as *Bacillus anthracis* protective antigen (PA), those protective against *Bordetella pertussis* such as *Bordetella pertussis* P69 antigen, those protective against *Schistoma mansoni* such as *Schistoma mansoni* glutathione-S-transferase, those protective against cholera such as Fibrio cholera β sub-unit, those protective against Herpes simplex virus(HSV) such as HSV glycoprotein D, those protective against HIV infection such as HIV envelope protein, and those protective against *Escherischia coli* such as *E. coli* LTB subunit or *E. coli* K88 antigen. Other suitable antigenic agents as those protective against *Mycrobacterium tuberculosis* as well as agents which protects or enhances anti-tumour immunity. In particular, it has been found that where the heterologous protein, is able to induce a protective immune response against *Yersinia pestis*, useful protective immunity is found. Examples of antigens which can produce such as response include the F1-antigen of *Yersinia pestis* or an antigenic fragment or variant thereof, or the V-antigen of *Yersinia pestis* or combinations thereof as described in WO 96/28551.

The expression "variant" refers to sequences of amino acids which differ from the base sequence from which they are derived in that one or more amino acids within the sequence are substituted for other amino acids. Amino acid substitutions may

be regarded as "conservative" where an amino acid is replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type. Broadly speaking, fewer non-conservative substitutions will be possible without altering the biological activity of the polypeptide. Suitably variants will be at least 60% homologous, preferably at least 75% homologous, and more preferably at least 90% homologous to the base sequence. Homology in this instance can be determined using in particular the Needleman-Wunsch algorithm with gap penalty of 8 using a standard PAM scoring matrix (Needleman S.B. and Wunsch C.D., J. Mol Biol. 1970, vol 48, 443-453).

The recombinant gut-colonising microorganisms described above are thus particularly suitable for use in the preparation of vaccines for therapeutic or prophylactic purposes, where they may be combined with a pharmaceutically acceptable carrier or diluent, as would be understood in the art.

In particular, the vaccines will be formulated so that they are adapted for oral administration and that the microorganism remains viable throughout any storage period. Thus they may preferably be in a form liquid form such as aqueous or oily suspensions, emulsions, syrups or elixirs.

The size of the dose for therapeutic or prophylactic purposes of will vary according to a wide variety of factors including the nature of the protective immune response sought, the nature of the antigen being employed, the severity of the conditions, the dosage regime in terms of primary and secondary boosting, the age and sex of the animal or patient and the gut-colonising ability of the particular microorganism used. In general however, a dosage of microorganism in the range of from 10^6 to 10^9 cfu will be administered as a single dosage.

Vaccine compositions may further comprise a buffer such as a bicarbonate buffer, in order to neutralise stomach acid.

Thus in a further aspect, the invention provides a method of inducing a protective immune response against a pathogen in a mammal, said method comprising administering to said mammal a recombinant gut-colonising microorganism which comprises a promoter selected from the P_{ompC}, P_{phoP} and P_{pagC} or fragments or variants thereof which can act as promoters, said promoter being operatively interconnected with a nucleic acid which encodes an antigen protein, able to induce a protective immune response against said pathogen, in a mammal to which it is administered.

In yet a further aspect, the invention provides the use of a promoter selected from P_{ompC}, P_{phoP} and P_{pagC} in the production of a vaccine comprising a recombinant gut-colonising organism.

The promoters used in this study are induced at specific sites in the body. They are preferably cloned into the microorganism in a low copy number vector, because high copy number plasmids have been shown to be unstable in *S typhimurium* (Coulson N M, et al., Microb Pathog. 1994;16:305-11).
The PhoP gene would be expected to be expressed at a basal level from the *PhoPp2* promoter and upregulated in the phagosome of host cells as a result of activation of the *PhoPp1* promoter (Soncini F C, et al., J. Bacteriol. 1995;177:4364-71). The PhoP / PhoQ regulatory system has been shown to regulate the expression of a variety of genes including *pagC*, and to be important for survival in macrophages (Miller S I. et al., Proc. Natl. Acad, Sci USA 1989;86:5054-58, Wick M J, et al., Mol. Microbiol. 1995;16:465-76).

Genes regulated by the PhoP/PhoQ system are also important for the virulence of orally delivered bacteria (Galan J E, et al., Microb Pathog. 1989;6:433-43). Expression of the *ompC* is gene is upregulated under conditions of high osmotic strength (Foster J

W, et al., Microbiology 1994;140:341-52, Nikaido H, et al., Cellular and Molecular Biology. American Society for Microbiology, Washington DC. 1987, pp7-22), such as those found within the gut, under control of the OmpR/EnvZ regulatory system (Pratt L A, et al., American Society for Microbiology, ASM Press, Washington DC, 1995, pp105-27).

Whilst the different plasmids in *S. typhimurium* SL3261 were stable in vitro, there were marked differences in the stability of the plasmids in bacteria which had been delivered to mice by the oral route. Bacteria expressing the F1-antigen from the *pagC* promoter showed a much reduced ability to colonise mesenteric lymph nodes and appeared incapable of further invasion of the host. It is possible that the additional copies of the *pagC* promoter and upstream regulatory regions titrated out the available PhoP activator within the cell, and that this prevented the bacterium from responding to the environmental changes encountered after uptake by M-cells. However, recombinant *Salmonella* containing the P_{phoP}-F1 plasmid did not show a similar inability to invade the host.

This finding might be in accordance with the suggestion that *phoP* expression is only partially autoregulated by the *phoP* gene product (Fields P I, et al., Science 1989;243:1059-62). Additionally, it is possible that the high level of expression of F1-antigen from the *pagC* promoter in vivo placed a lethal metabolic load on the host bacterium.

These promoters are regulated by a variety of environmental stimuli in a manner which is not fully defined. Therefore, it is difficult to make meaningful comparisons of the strengths of these promoters in vitro. Thus, *in vivo* testing of these promoters to identify those most suitable for use for the expression of guest antigens has been carried out.

All of the recombinant *Salmonella* induced similar levels of antibody against the whole bacterium. This finding was unexpected for bacteria containing pP_{pagC}-F1, since these bacteria were unable to invade deep host tissues and were recovered only at low levels from mesenteric lymph nodes. This recombinant *Salmonella* also induced IgG and IgA antibody against the F1-antigen. This suggests that the initial interaction of the bacteria with M-cells is critical in determining the immune response to the bacterium and to guest antigens. This conclusion is supported by the finding that *Salmonella* containing pP_{pagC}-F1 induced mucosal antibody to the F1-antigen whereas bacteria expressing the F1-antigen expressed from the *lacZ* or *ompC* promoters failed to induce mucosal responses. Therefore, the measurement of the colonisation of spleen or liver tissues, as an indicator of vaccine potential of recombinant *Salmonella*, may not always be useful.

Similar conclusions were reached by Covone et al. (Covone M G, et al., Infect. Immun. 1998;66:224-31) who showed that effective delivery of the LTK63 guest antigen to the immune system was effective only when the antigen was delivered during the early stages of invasion and by McSorley et al. (McSorley S J, et al., Infect. Immun. 1997;65:171-78) who showed that recombinant *Salmonella* expressing glycoprotein 63 from the *osmC* promoter were unable to invade tissue beyond the mediastinal lymph nodes, yet induced protection against *Leishmania major*. This might also explain why killed *Salmonella* with or without guest antigens, which are clearly not able to invade deep host tissues, are able to induce an immune response (Thatte J, et al., Int. Immunol. 1993;5:1431-36).

The ability of *Salmonella* expressing P_{phoP}-F1 to induce mucosal antibody responses to the F1-antigen in both the gut and the lungs, whereas a constitutive promoter (P_{lacZ}) failed to induce such responses clearly demonstrates the utility of *in vivo* induced promoters for the induction of appropriate antibody

responses. This promoter system will be particularly useful for other applications where a mucosal antibody response is important for protection against disease.

The invention will now be particularly described by way of Example with reference to the accompanying diagrammatic drawings in which:

Figure 1 is a plasmid diagram illustrating plasmids used in the preparation of microorganisms in accordance with the invention;

Figure 2 is a graph showing the levels of colonisation of spleen tissues of mice, 11 days after dosing with recombinant microorganisms of the invention;

Figure 3 shows graphs illustrating IgG serum antibody levels in mice to the carrier bacterium, (Fig 3a) and to the F1 antigen (Fig 3b), 21, 28 and 98 days after immunisation;

Figure 4 is a graph showing the isotype of the F1 antibody found in mice serum on day 98, where the blank column represents the amount of the $IgG1_a$? and the shaded column represents the $IgG2_a$ isotype in all groups of immunised animals:

Figure 5 is a graph showing the levels of circulating IgA antibody to F1-antigen or the levels of IgA antibody to F1-antigen in gut (blank column) or lung (shaded column) wash samples;

Figure 6 shows sequences of promoters used in the evaluation of the invention; and

Figure 7 shows the results of elispot analysis of Peyer's patch cells and in particular the IgA response against F1 antigen (Figure 7a) and Salmonella (Figure 7b).

Example 1

Preparation of Bacterial strains, cultivation and enzymes

Escherichia coli strain JM109 and *S. typhimurium* strains LB5010 (rm* *galE*), SL3261 (*aroA*) or SL1344 (a mouse-virulent strain; (Zhang X, et al., Infect. Immun. 1997;65:5381-7) were cultured on L-agar or in L-broth, supplemented with ampicillin (05µg/ml) where appropriate. Enzymes used for DNA cloning and amplification procedures were obtained from BCL limited (Lewes, Sussex, UK). PCR reactions were carried out using a Perkin Elmer 9600 (P.E. Applied Biosystems, Warrington, UK) thermal cycler with cycle conditions of 95°C, 5 min, followed by 50 cycles of 95°C, 5s; 45°C, 5s; 72°C, 5s, followed by 10 min at 72°C.

Plasmids containing promoters for expression of F1-antigen were then produced. The promoters for the *phoP*, *pagC* and *ompC* genes have previously been mapped and upstream regulatory regions identified (Soncini F C, et al., J. Bacteriol. 1995;177:4364-71, Pulkkinen W S, et al., J. Bacteriol. 1991;173:86-9, Puente J L, et al., Gene. 1987;61:75-83, Puente J L, et al., Gene. 1989;83:197-206). For the *phoP* gene promoter a 139bp DNA fragment was identified which included the *phoPp1* and *phoPp2* gene promoters and 80 bp upstream of the -35 site which has been predicted to form step loop structures (Soncini F C, et al., J. Bacteriol. 1995;177:4364-71). For the *pagC* gene promote a 715 bp DNA fragment included 125 bp upstream of the -35 region (Pulkkinen W S, et al., J. Bacteriol. 1991;173:86-9). For the *ompC* gene promoter a 371 bp DNA fragment included a 275 bp region upstream of the -35 region (Puente J L, et al., Gene. 1987;61:75-83, Puente J L, et al., Gene. 1989;83:197-206).

These DNA fragments were amplified from *S. typhimurium* strain SL1344 genomic DNA using the PCR. For comparison with a constitutive gene promoter, a 196 bp DNA fragment encoding the *lacZ* gene promoter and 140 bp upstream of the -35 region was identified. The 3′ end of all of the DNA fragments terminated

before the SD regions associated with the genes. These promoters were cloned upstream of the *caf1* open reading frame (encoding the *Y. pestis* F1-antigen) and SD region in a low copy number vector (pBR322; Fig 1) and the recombinant plasmids (pP_{ompC}-F1, pP_{phop}-F1, pP_{pagC}-F1 or pP_{lacZ}-F1) transformed into *S. typhimurium* SL3261 (*aro*A).

Oligonucleotide primers were designed to amplify promoter regions using the PCR (Table 1).

Table 1

Oligonucleotide pair	SEQ ID No	Prom
5'AAGGAAAAAAGCGGCCGCCGCCCAATACGCAAACCG 3'	5	
5'GAATTCACTAGTATTGTTATCCGCGCTCACAAT 3'	6	*Plac*
5'AAGGAAAAAAGCGGCCGCTGACTCTGGTCGACGAACTTA 3'	7	
5'CTAGTCTAGATGTGTTAACCAATAAGAACAGTCTA 3'	8	*PphoP*
5'AAGGAAAAAAGCGGCCGCTAAACAGACATTCAGAAGTGAATG3'	9	
5'CTAGTCTAGAATATGCCTTTATTGCTTTTTTATG 3'	10	*PompC*
5'AAGGAAAAAAGCGGCCGCGTTAACCACTCTTAATAATAATG 3'	11	
5'CTAGACTAGTTACTACTTATTATTTACG 3'	12	*PpagC*

Restriction sites are shown underlined.

The primers included unique *Not*1, *Xba*l or *Spe*l sites. Regions amplified included the -10 and -35 regions and upstream regulatory binding sites, but excluded the Shine-Dalgarno (SD) ribosome binding site.

After PCR amplification of the promoter regions from *S. typhimurium* SL 1344 template DNA (or plasmid pUC18 template DNA for amplification of the *lac* promoter), the DNA fragments were purified using Microcon 100 centrifugal concentrations (Millipore, Watford, UK). The purified DNA fragments were cloned into suitable digested plasmid pBluescript SK-,

electroporated into *E. coli* JM101 and the cloned fragments were nucleotide sequenced to ensure their authenticity. After digestion of the recombinant plasmids with *Sac*I and *Bss*HII and agarose gel electrophoresis, DNA fragments containing the promoter regions were purified using Qiaex (Qiagen Ltd, Crawley, UK) and blunt ended using Klenow fragment (Sambrook J, Frtisch E F, Maniatis T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory press, New York).

The authentic promoter sequences were then cloned into plasmid pBR322 which had been digested with *Eco*RI and *Nru*I and then blunt ended using Klenow fragment (Sambrook J, Frtisch E F, Maniatis T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory press, New York). The orientation of the cloned DNA fragment in the plasmid was determined by analysing, using agarose gel electrophoresis, the DNA fragments obtained after digestion with *Xba*I, *Ssp*I or *Sty*I.

A DNA fragment which encoded the Caf1 open reading frame and the ribosome binding site was isolated after digestion of plasmid pORF1 (Oyston P C F, et al., Infect. Immun. 1995;63:563-68) with *Eco*RI followed by blunt ending of the DNA and further digestion with *Hind*III. The purified DNA fragment was ligated with promoter plasmids with which had been digested with *Sma*I and *Hind*III. The final recombinant plasmids were transformed into *E. coli* strain JM109.

Plasmids were isolated from *E. coli* (Sambrook J, Frtisch E F, Maniatis T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory press, New York) and electroporated into *S. typhimurium* SL3281 (*aro*A) after passage through *S. typhimurium* LB5010 to ensure methylation of the DNA.

Example 2

The stability of the different plasmids encoding F1-antigen driven from different promoters in *S. typhimurium* SL3261 was

determined after culture of the bacteria in L-broth for 24hr (*in vitro* stability) and enumeration of bacteria which grew on L-agar or L-agar containing amplicillin (Sambrook J, Frtisch E F, Maniatis T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory press, New York). The results indicated that all of the plasmids were retained by at least 80% of the bacteria which had been cultured *in vitro* (pP_{ompC}-F1, 83%; pP_{phoP}-F1, 100%; pP_{pagC}-F1, 98%; pP_{lacZ}-F1, 95%).

Example 3

Colonisation of host tissues

The *in vivo* stability of plasmids was determined by inoculating groups of 10 female BALB/c mice orally with 10^9 cfu of bacteria in 100µl of PBS, and enumerating bacteria isolated 11 days later from homogenised spleen tissue on L-agar or L-agar + ampicillin.

In the case of the P_{pagC}-F1 construct and the *S. typhimurium* SL3261 control strain, bacteria were also cultured from mesenteric lymph nodes (10/mouse) each homogenised in 2ml of PBS or from homogenised liver tissue which were removed on 11 days after dosing. Bacteria were enumerated bacteria as described above.

With the exception of the bacteria containing the P_{pagC}-F1 plasmid and the SL3261 control bacteria, ampicillin-resistant bacteria could be recovered from all of the spleens isolated from orally dosed mice. The *in vivo* stability of all of the plasmids within *Salmonella* was lower than the stability of the plasmids within *Salmonella* cultured *in vitro*.

Although mice were dosed orally with similar numbers of bacteria there were marked differences in the level of colonisation of spleen tissues at day 11 (Fig 2). The highest levels of colonisation were found with bacteria containing pP_{phoP}-F1. Low numbers of *Salmonella* containing pP_{pagC}-F1 were recovered from spleen tissues (maximum 50 cfu/spleen) and none of the bacterial

colonies were ampicillin resistant. To investigate why the presence of the pP_{pagC}-F1 affected the colonising ability of this *Salmonella*, a more detailed study was undertaken which involved the enumeration of bacteria in the liver and in mesenteric lymph nodes 11 days after oral dosing. The levels of SL3261 containing pP_{pagC}-F1 isolated from liver tissues were similar to those isolated from spleens (data not shown). Whereas the mean number of SL3261 isolated from mesenteric lymph nodes was $2.2x10^3$ cfu, SL3261 containing pP_{pagC}-F1 could be isolated only at low level (mean 20 cfu) from these tissues.

Example 4

Serum antibody responses to F1-antigen

Groups of 5 or 8 female BALB/c mice were immunised via intragastric intubation on days 0 and 14 with 1 x 10^9 cells of the P_{ompC}-F1, P_{phoP}-F1, P_{pagC}-F1 or P_{lacZ}-F1 constructs, or the control *S. typhimurium* strain, SL3261, in 0.1 ml of phosphate-buffered saline (PBS). Bacteria were grown statically overnight at 37°C. All oral inoculations were carried out with a stainless steel gavage needle without an anaesthetic. The inoculum dose was verified by plating serial dilutions of each culture on L-agar plates with or without ampicillin.

On days 21, 28 and 98 mice were anaesthetized by intraperitoneal (i.p.) administration of a cocktail of domitor (6 mg per dose) and Ketalar (27mg per dose) and blood was collected by cardiac puncture. Mice were then sacrificed by cervical dislocation. Blood was allowed to clot at 4°C overnight prior to centrifugation (10,000 x g, 10 min, 4°C) and the serum stored at -20°C until tested.

After i.g. dosing with the recombinant *Salmonella*, mice in all groups developed IgG serum antibody to the carrier bacterium, which reached a maximum level 98 days after immunisation (Fig 3a). In the case of SL3261 expressing P_{phoP}-F1, the onset of the immune response was delayed. All groups of mice developed IgG

serum antibody to F1-antigen (Fig 3b), which had reached a peak at day 28 and declined by day 98. There was no significant difference in the peak titres to F1-antigen induced by the different recombinant *Salmonella*.

When the isotype of this antibody was determined on day 98, it was found to be predominantly of an $IgG2_a$ isotype in all groups of immunised animals (Fig 4), suggesting that a Th1-type response was induced. Several other workers have shown that recombinant S. typhimurium induce a Th1-type response to the guest antigen (Brett S J, et al., Immunology 1993;80:306-12, Thatte J, et al., Int. Immunol. 1993;5:1431-36).

Example 5

Mucosal antibody responses to F1-antigen

The ability of the different recombinant *Salmonella* to induce a mucosal antibody response after i.g. dosing was determined by measuring the levels of circulating IgA antibody to F1-antigen or the levels of IgA antibody to F1-antigen in gut or lung wash samples. After dosing as described in Example 4, on days 21 and 28, gut and lung wash samples were collected. Briefly, gut wash samples were collected by resecting a 10 cm length of small intestine and flushing with 5 ml of PBS. Samples were sonicated for 0.5 min prior to centrifugation (12,000 x g, 30 mins 4°C) and the supernatant was decanted and lyophilized. Broncho-alveolar washings were collected from individual animals by injecting 5 ml of chilled lavage medium (0.9% (w/v) NaCl, 0.05% (v/v) tween 20, 0.1% (w/v) NaN_3 and 1mM phenylmethylsulfonyl fluoride) into the trachea using an intravenous canula and inflating the lungs. A syringe was used to remove the washings, which were subsequently centrifuged (12,000 x g, 30 min, 4°C) prior to lyophilisation of the supernatant fluid. Gut and lung wash samples were reconstituted in 200µl sterile water immediately before use.

All measurements of antibody levels in individual animals were determined in duplicate. For enzyme-linked immunosorbant assays (ELISAs) to determine IgG and IgA titres, 96-well microtiter plates were coated overnight at 4°C either with 50µl 5µg/ml purified F1-antigen (Miller J, et al., FEMS Microbiology and Immunology 1998;21:213-21) in PBS or with 50µl 6µg/ml *S. typhimurium* SL3261 lysate in PBS, prepared as follows. Bacteria were grown statically overnight at 37°C, prior to harvesting and resuspension in PBS to an approximate concentration of 1 x 10^{10} cfu/ml. Cells were heat-killed in a boiling water bath for 30 min, cooled on ice and then sonicated on ice for 6 pulses of 30 s. Total protein concentration was determined by a BCA protein assay (Pierce and Warriner, Chester, UK). Plates were blocked for 1 h at 37°C with PBS containing 1% (w/v) skimmed milk powder (BLOTTO). Serum, gut and lung wash samples were diluted in BLOTTO and 50µl volumes were assayed in duplicate in a series of twofold dilutions. After incubation overnight at 4°C, plates were washed three times in PBS with 0.02% (v/v) tween 20. Peroxidase-conjugated secondary antibodies against mouse IgG or IgA (Harlan Sera-Lab Ltd, Loughborough, UK), diluted 1:2000 in BLOTTO were incubated for 1 h at 37°C. The plate was washed as previously and 100µl of 2,2'-azino bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (ABTS; Sigma, Poole, UK) was added. Antibody titre was estimated as the maximum dilution of serum giving an $absorbance_{414nm}$ reading 0.1 U above background (Sera from animals immunised with SL3261 alone).

To determine IgG_1, or IgG_{2a} concentrations, ELISAs were performed essentially as above, except that wells were coated with 10µg/ml anti-mouse IgG (Fab-specific, Sigma, Poole, UK) 5µg/ml purified F1-antigen in PBS or 6µg/ml S. typhimurium SL3261 lysate. Purified IgG_1 or IgG_{2a} (Sigma, Poole, UK) and day 98 serum samples were diluted in BLOTTO. Peroxidase-labelled secondary antibodies against mouse IgG_1 or IgG_{2a} were diluted 1:4000 BLOTTO before use.

The results (Fig 5) indicated that SL3261 containing pP_{phoP}-F1, pP_{lacZ}-F1 or pP_{pagC}-F1 plasmids all induced serum IgA antibody to F-antigen. The induction of circulating IgA to F1-antigen did not correlate with the presence of IgA to F1-antigen at mucosal surfaces. For example, SL3261 / pP_{lacZ}-F1 induced high levels of serum antibody to F1-antigen but IgA antibody to F1-antigen was not detected in gut or lung wash samples. Only SL3261 / pP_{phoP}-F1 induced an IgA antibody response to F1-antigen in both the gut and the lung.

Example 6

Production of IgA by Peyer's patch cells

Peyer's patches were also removed to determine the presence of F1- and Salmonella specific IgA producing cells in the gut. Briefly, a total of 8 Peyer's patches were removed from 5 mice in each treatment group (see Example 4) and pooled. Cells were separated by crushing through a cell strainer, washed by centrifugation and resuspended in 1.4ml of Dulbeccos Modified Eagles Medium (DMEM) + 10% foetal calf serum (FCS). Duplicate samples (100µl/well) were then plated onto plates previously coated with 5µl/ml F1 or 6µg/ml S. typhimurium SL3261 lysate and blocked with 20% FCS in DMEM, and incubated for 48hours at 37°C. Plates were washed and incubated for 1 hour with peroxidase-labelled secondary antibody against mouse IgA, diluted 1:2000 in PBS before use, and developed with 100µl of ABTS.

The results are shown in Figure 7. These show showed that the secretion of IgA against F1-antigen was greatest in Peyer's patch cells isolated from mice which had been immunised with SL3261 containing pP_{pagC}-F1 (Fig 7a). Peyer's patch cells taken from mice which had been immunised with SL3261 / pP_{phoP}-F1 also produced IgA. Cells taken from other groups produced only low levels of IgA to F1-antigen.

This pattern of response was not reflected in the pattern of production of IgA against *Salmonella*; Peyer's patch cells taken from mice which had been immunised with SL3261 / pP_{pagC}-F1 produced only low levels of antibody to *Salmonella* (Fig 7b).

Claims

1. A method of enhancing expression of a desired protein at mucosal effector sites, said method comprising placing the protein to be expressed under the control of a promoter having SEQ ID NO 2, SEQ ID NO 3 or SEQ ID NO 4 or a fragment or variant or any of these which has promoter activity, and causing expression in mucosal cells.

2. A construct comprising a promoter selected from the P_{ompC}, P_{phoP} and P_{pagC} or fragments or variants thereof which can act as promoters, operatively interconnected with a nucleic acid which encodes a protein, able to induce a protective immune response against an organism, in a mammal to which it is administered, wherein said construct contains no further elements of the ompC, phoP or pagC gene.

3. A recombinant gut-colonising microorganism which has been transformed with a construct according to claim 2.

4. A recombinant gut-colonising microorganism according to claim 3 wherein said protein is heterologous to said microorganism.

5. A recombinant gut-colonising microorganism according to claim 4 or claim 4 wherein the promoter is P_{phoP} promoter.

6. A recombinant gut-colonising microorganism according to claim 3 or claim 4 wherein the promoter is P_{pagC} promoter.

7. A recombinant gut-colonising microorganism according to any one of claims 3 to 6 which comprises a *Salmonella spp.*

8. A recombinant gut-colonising microorganism according to claim 7 wherein the *Salmonella spp.* is *Salmonella typhimurium* or *Salmonella typhi.*

9. A recombinant gut-colonising microorganism according to any one claims 3 to 8 wherein the gut-colonising microorganism is attenuated.

10. A construct according to claim 2 or a recombinant gut-colonising microorganism according to any one of claims 3 to 9 wherein the heterologous protein, is able to induce a protective immune response against *Yersinia pestis*.

11. A construct or a recombinant gut-colonising microrganism according to claim 10 wherein the said heterologous protein comprises an F1-antigen of *Yersinia pestis* or an antigenic fragment or variant thereof.

12. A vaccine comprising a recombinant gut-colonising microorganism according to any one claims 3 to 11.

13. A vaccine according to claim 12 which further comprises a pharmaceutically acceptable carrier or diluent.

14. A vaccine according to claim 12 or claim 13 which is adapted for oral administration.

15. A method of inducing a protective immune response against a pathogen in a mammal, said method comprising administering to said mammal a recombinant gut-colonising microorganism according to any one of claims 3 to 11.

16. The use of a promoter selected from P_{ompC}, P_{phoP} and P_{pagC} in the production of a vaccine comprising a recombinant gut-colonising organism.

Fig.1.

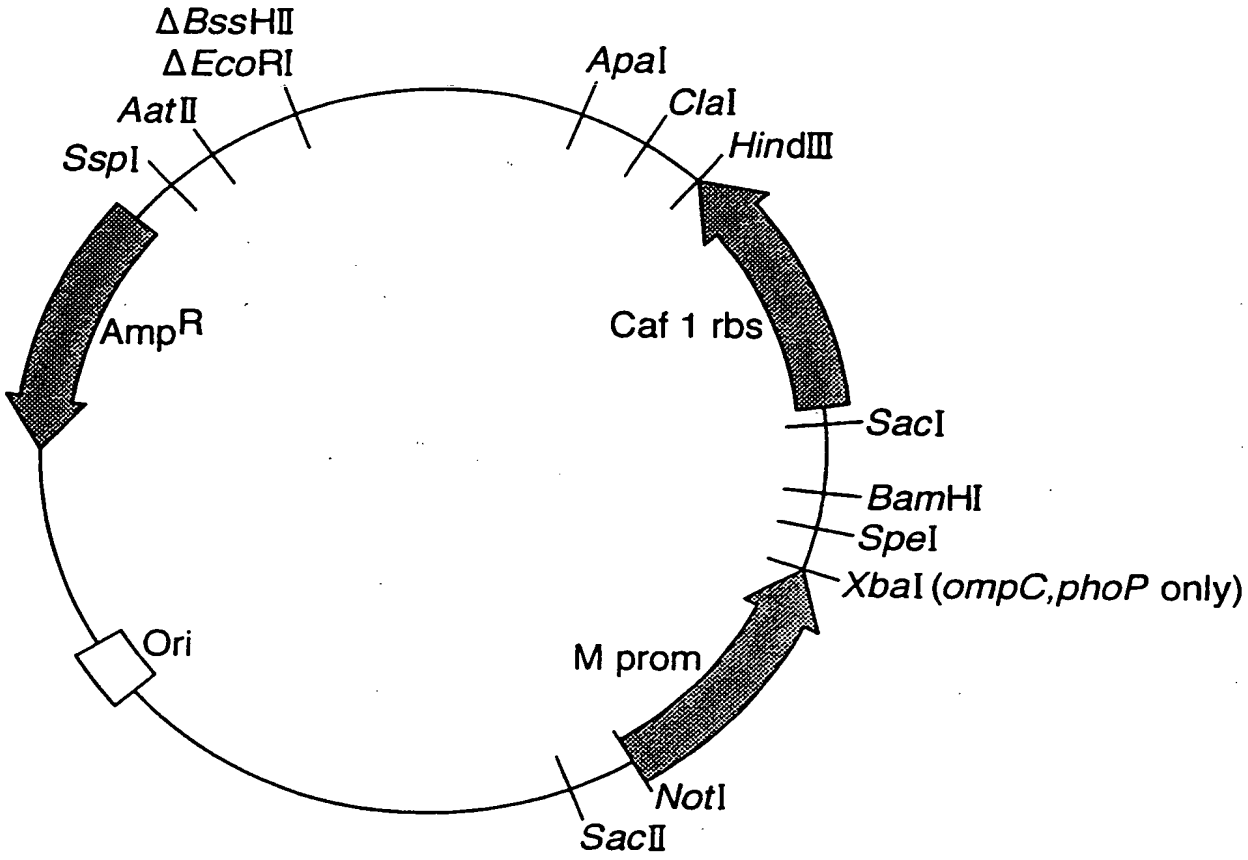
ΔBssHII
ΔEcoRI
AatII
SspI
ApaI
ClaI
HindIII
AmpR
Caf 1 rbs
SacI
BamHI
SpeI
XbaI (ompC,phoP only)
Ori
M prom
NotI
SacII

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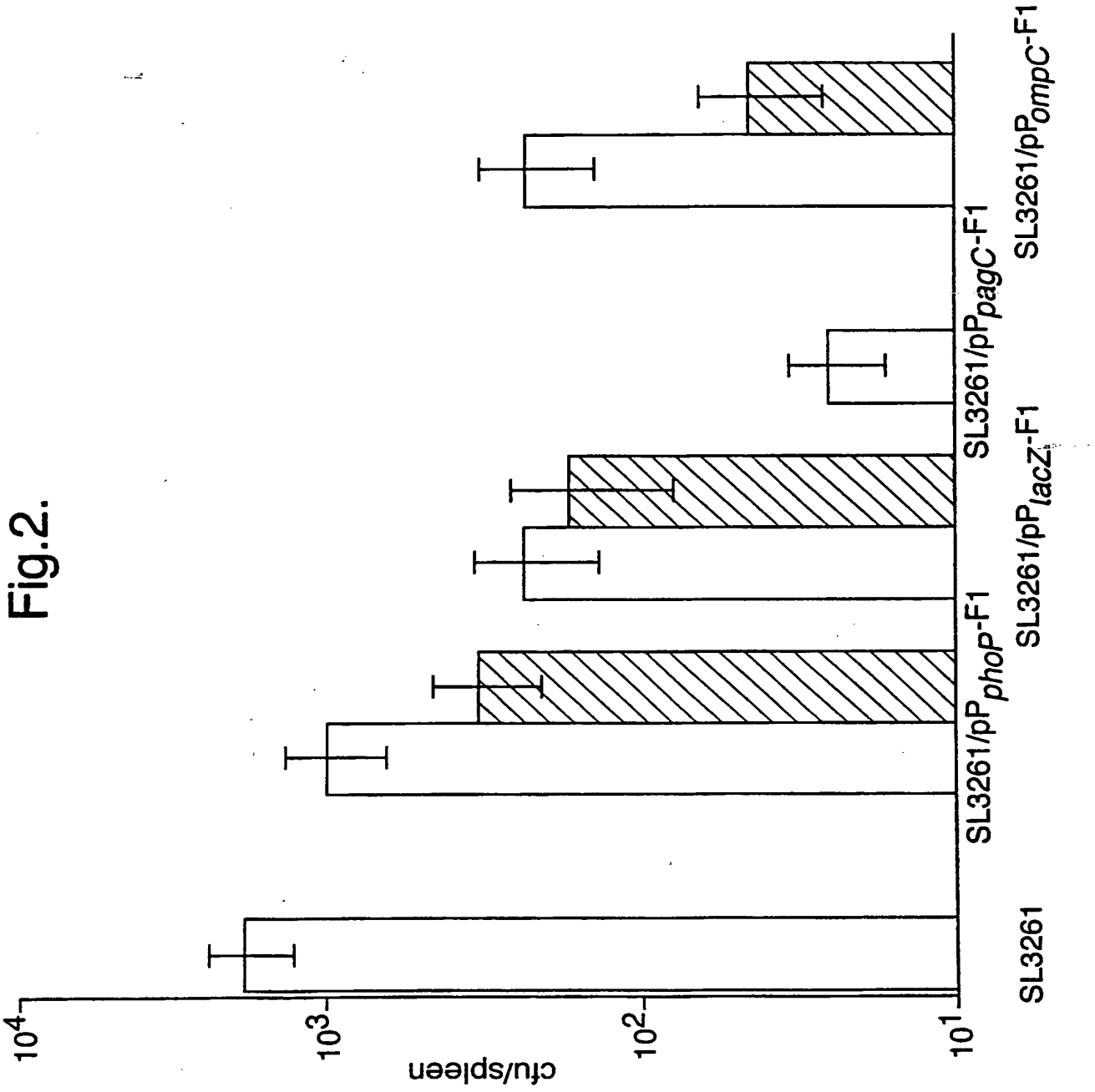
Fig.2.


cfu/spleen
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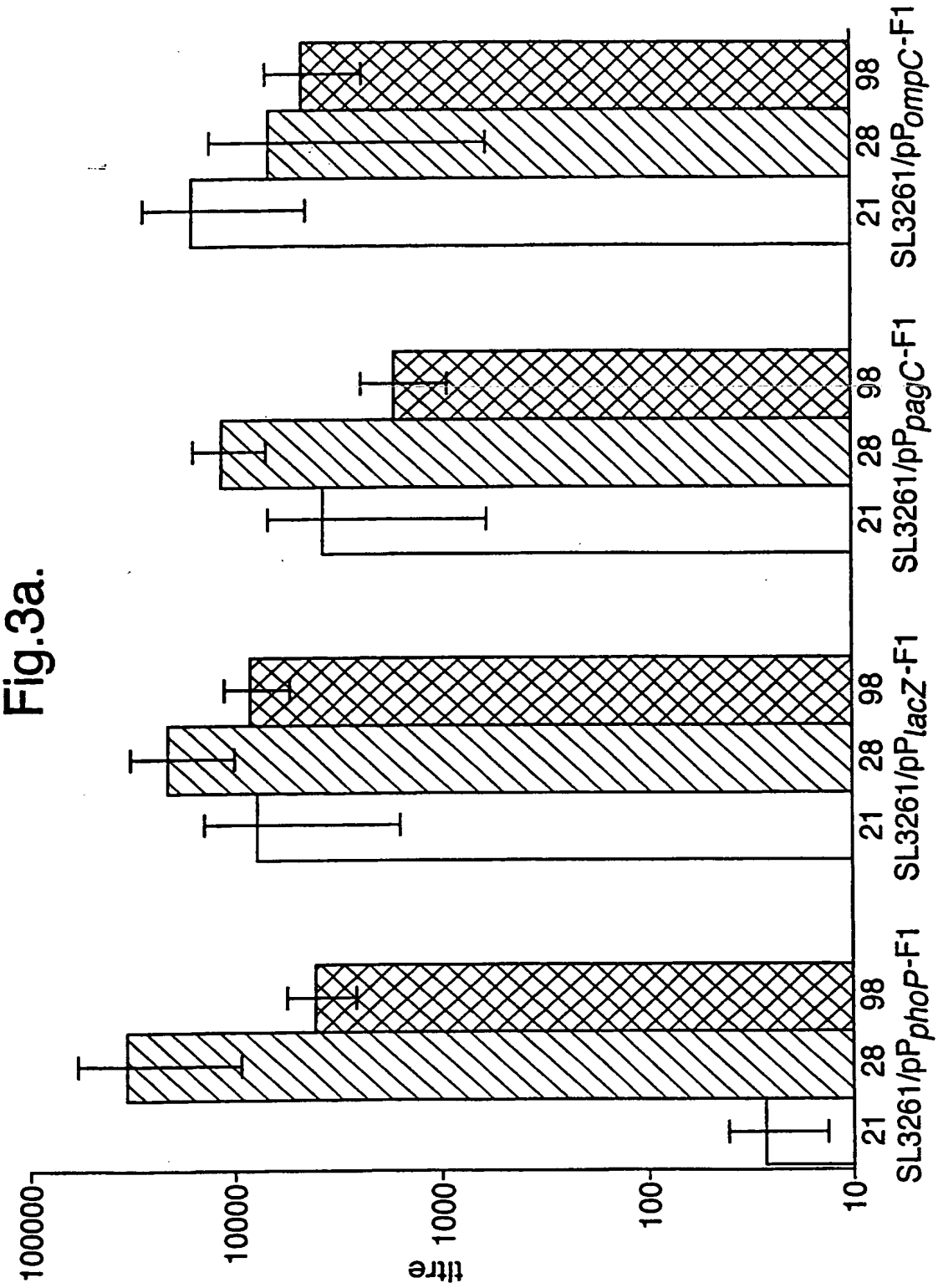
10^3
10^2
10^1
SL3261
SL3261/pP*phoP*-F1
SL3261/pP*lacZ*-F1
SL3261/pP*pagC*-F1
SL3261/pP*ompC*-F1

Fig.3a.


titre
100000
10000
1000
100
10
21
28
98
SL3261/pP*phoP*-F1
SL3261/pP*lacZ*-F1
SL3261/pP*pagC*-F1
SL3261/pP*ompC*-F1

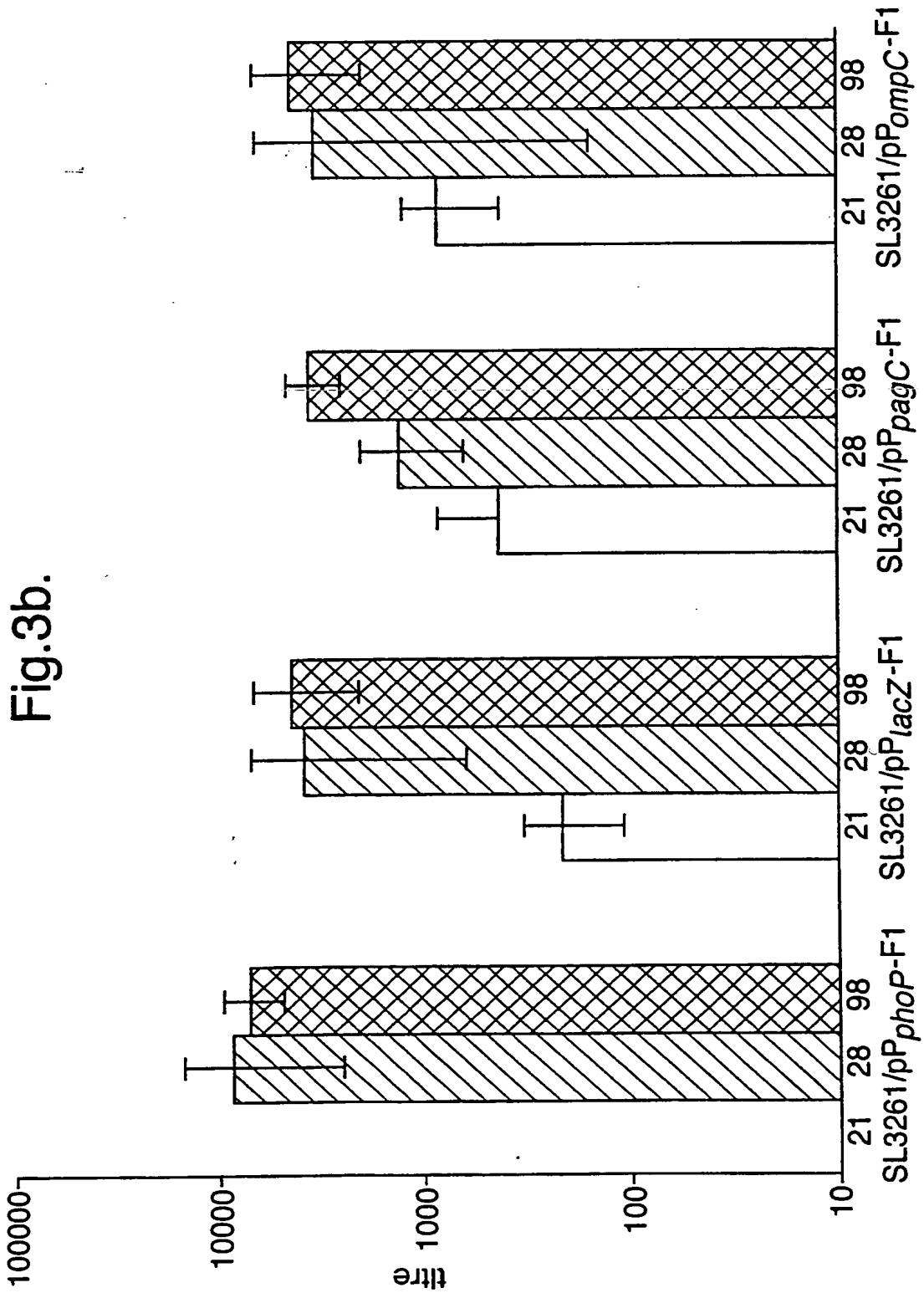
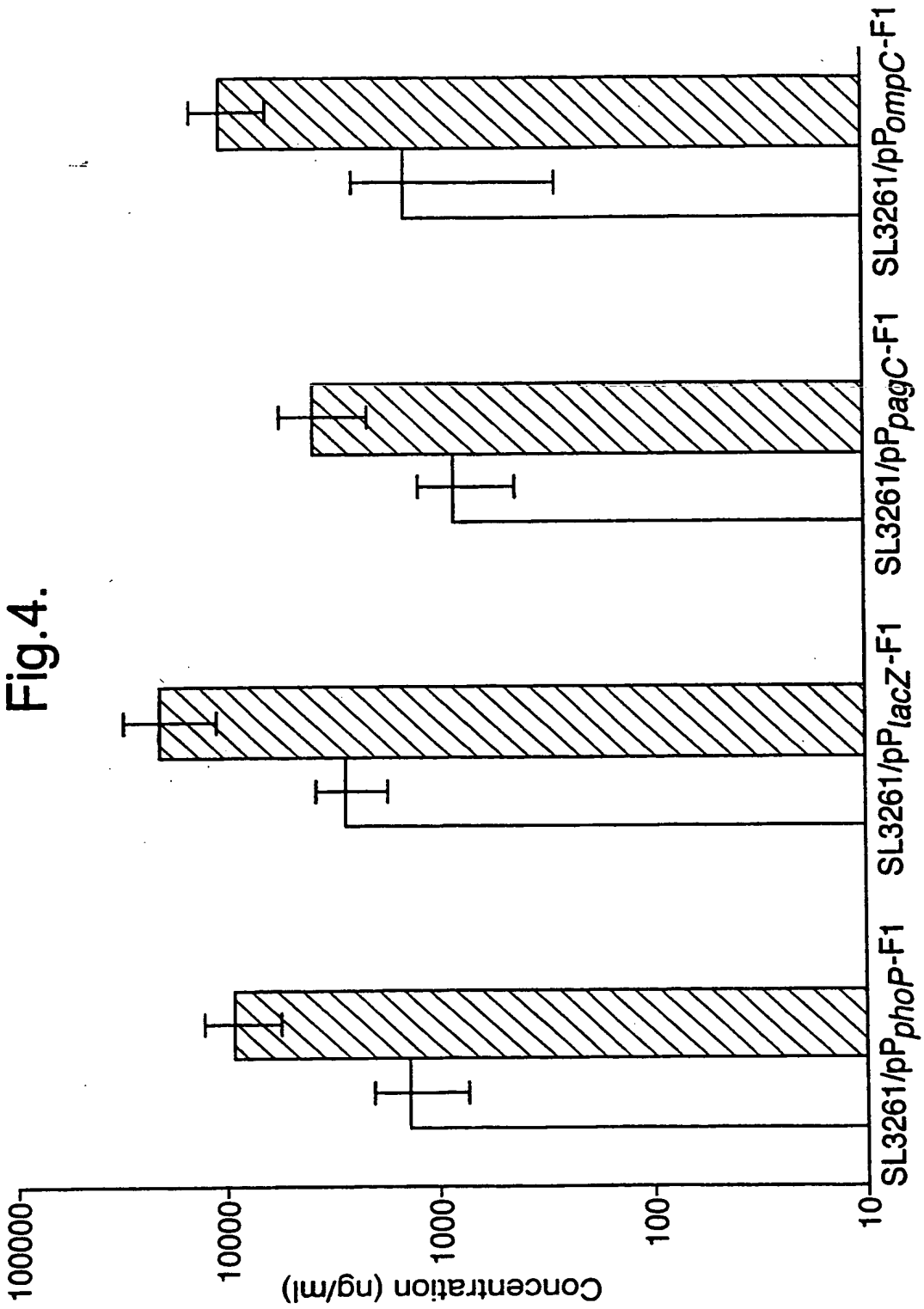

titre
100000
10000
1000
100
10
21
28
98
SL3261/pP*phoP*-F1
SL3261/pP*lacZ*-F1
SL3261/pP*pagC*-F1
SL3261/pP*ompC*-F1

Fig.3b.

Fig.4.


Concentration (ng/ml)
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10000
1000
100
10
SL3261/pP*phoP*-F1
SL3261/pP*lacZ*-F1
SL3261/pP*pagC*-F1
SL3261/pP*ompC*-F1

Fig.5.

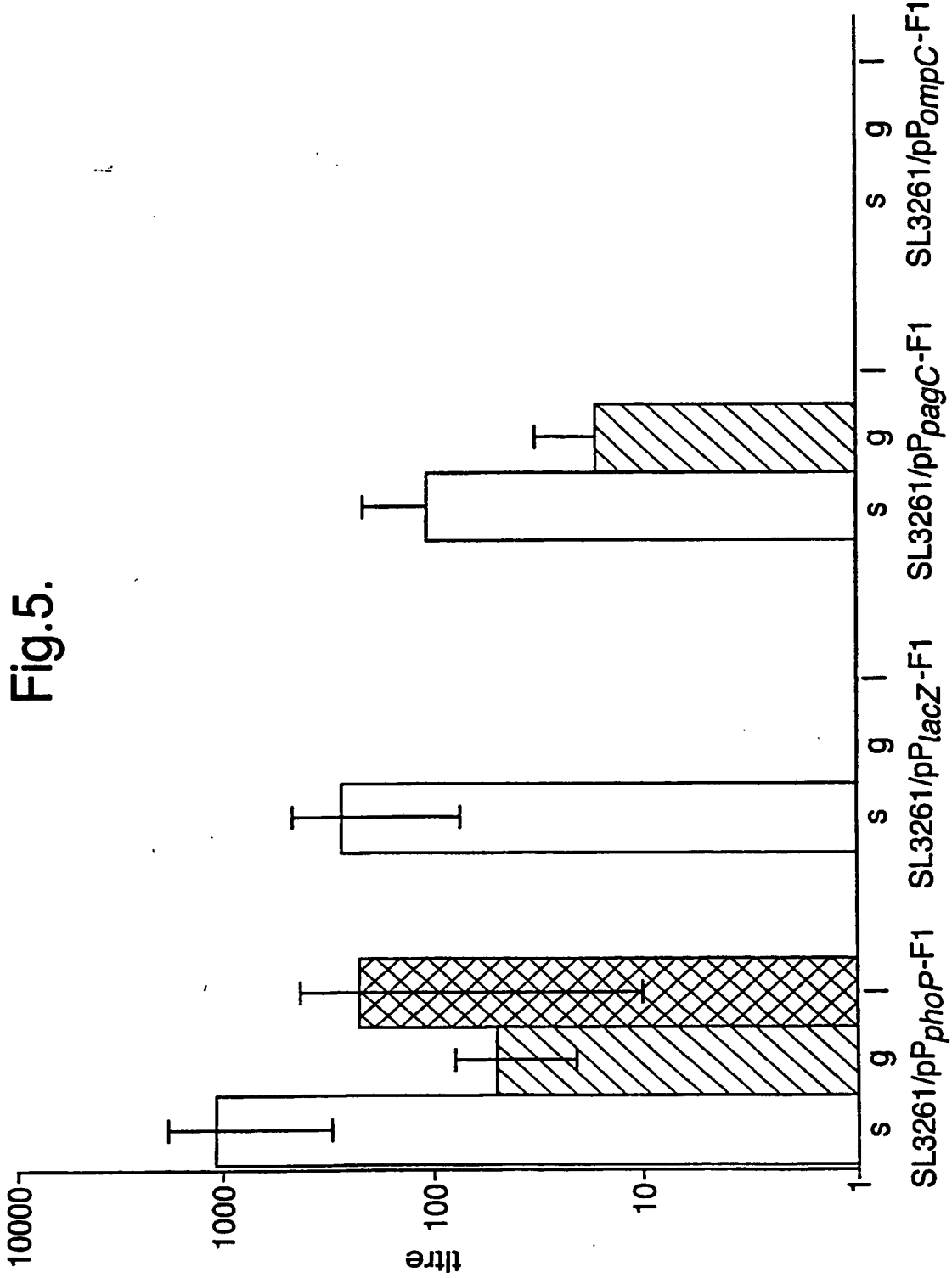

titre
10000
1000
100
10
1
s
g
l
SL3261/pP*phoP*-F1
SL3261/pP*lacZ*-F1
SL3261/pP*pagC*-F1
SL3261/pP*ompC*-F1

Fig.6.

P_{lac} 5'

CGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCC

CGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTT

TACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAAT 3'

(SEQ ID NO 1)

P_{phoP} 5'

GTGACTCTGGTCGACGAACTTAAATAATGCCTGCCTCACCCTCTTTTCTTCAGAAAGAGGGTGACTATTTG

TCTGGTTTATTAACTGTTTATCCCCAAAGCACCATAATCAACGCTAGACTGTTCTTATTGTTAACACA 3'

(SEQ ID NO 2)

P_{pagC} 5'

GTTAACCACTCTTAATAATAATGGGTTTTATAGCGAAATAGACTTTTTTATCGCGTGTTCAATATTTGCGT

TAGTTATTATTTTTTTGGAATGTAAATTCTCTCTAAACACAGGTGATATTTATGTTGGAATTGTGGTGTTG

ATTCTATTCTTATAATATAACAAGAAATGTTGTAACTGATAGATATATTAAAAGATTAAATCGGAGCGGGA

ATAAAGCGTGCTAAGCATCATCGTGAATATGATTACAGCGCCTGCGATGGCATATAACCGTATTGCGGATG

GAGCGTCACGTGAGGACTGTGAAGCACAATGCGATATGTTCTGATTATATGGCGAGTTTGCTTAATGACAT

GTTTTTAGCCGAACGGTGTCAAGTTTCTTAATGTGGTTGTGAGATTTTCTCTTTAAATATCAAAATGTTGC

ATGGGTGATTTGTTGTTCTATAGTGGCTAAACACTTTATGGTTTCTGTTAAATATATATGCGTGAGAAAAA

TTAGCATTCAAATCTATAAAAGTTAGATGACATTGTAGAACCGGTTACCTAAATGAGCGATAGAGTGCTTC

GGTAGTAAAAATATCTTTCAGGAAGTAAACACATCAGGAGCGATAGCGGTGAATTATTCGTGGTTTTGTCG

ATTCGGCATAGTGGCGATAACTGAATGCCGGATCGGTACTGCAGGTGTTTAAACACACCGTAAATAATAAG

TAGTA 3' (SEQ ID NO 3)

P_{ompC} 5'

TAAACAGACATTCAGAAGTGAATGACGGTAATAAATAAAGTTAATGATGATAGCGGTCACTATTTTAGTTG

CGAATGAAGATTCTGTTTTATCATTCAGTGCTATGAATTTCATCAATTTAACCCGTTGATTTTAAAAGTTT

CGTGAAATATATTTTGTCTATTTGTGCTTATTTTTACTTGATTTTTGCTTTAAAAAAGTTCCGTAAAATTC

ATATTTTGAAACATCTATATAGATAACTGTAACATCTTAAAAGTTTTAGTATCATATTCGTGTTTGGATTAT

TCTGTATTTTTGCGGAGAATGGACTTGCCGACTGGTTAATGAGGGTTAACCAGTAAGCAGTGGCATAAAAA

AGCAATAAAGGCATAT 3'

The locations of oligonucleotide primers used in the PCR are shown underlined. Promoter regions are shown in bold and repeated sequences upstream of these promoters are shown double underlined

Fig.7a.

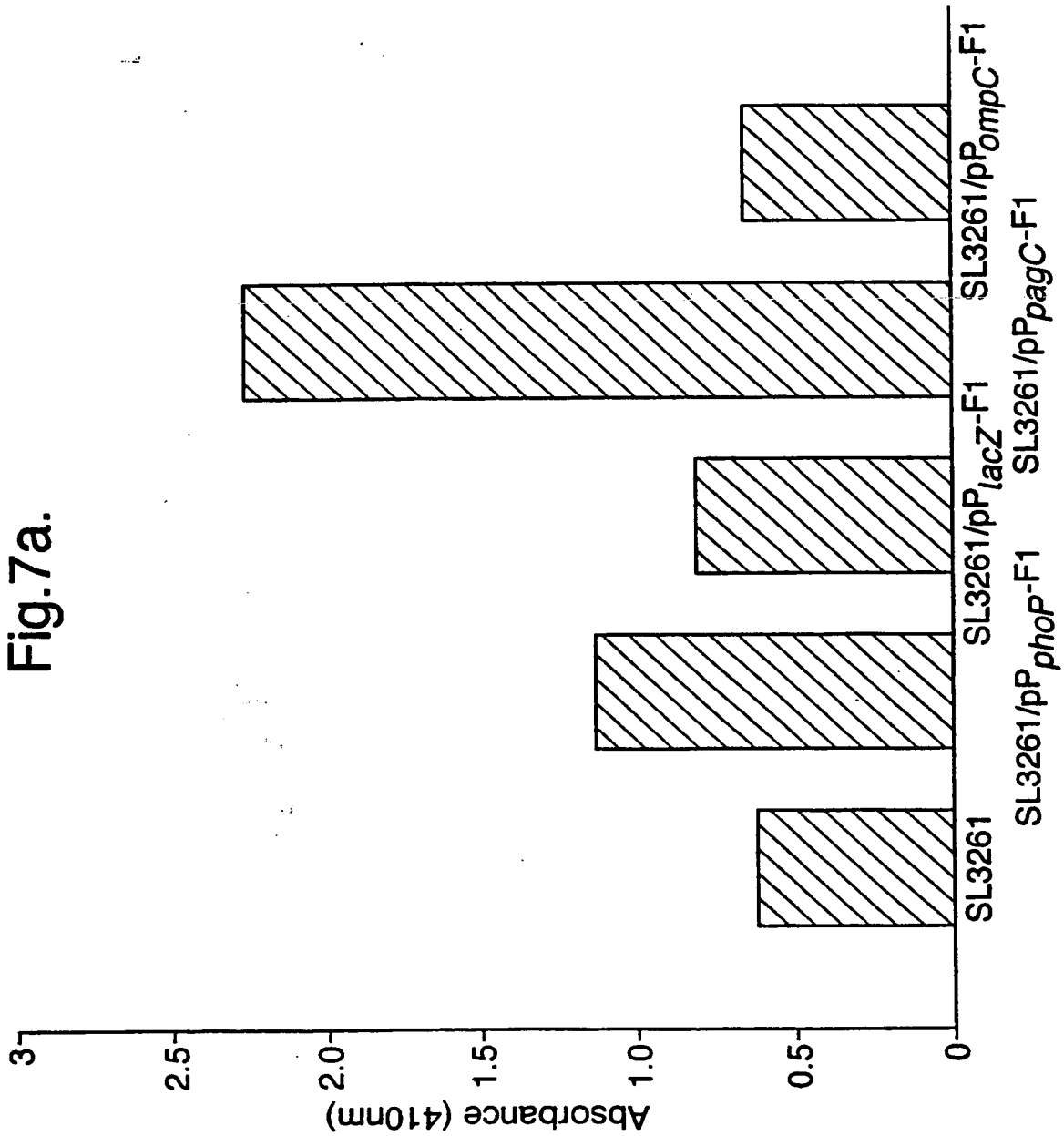
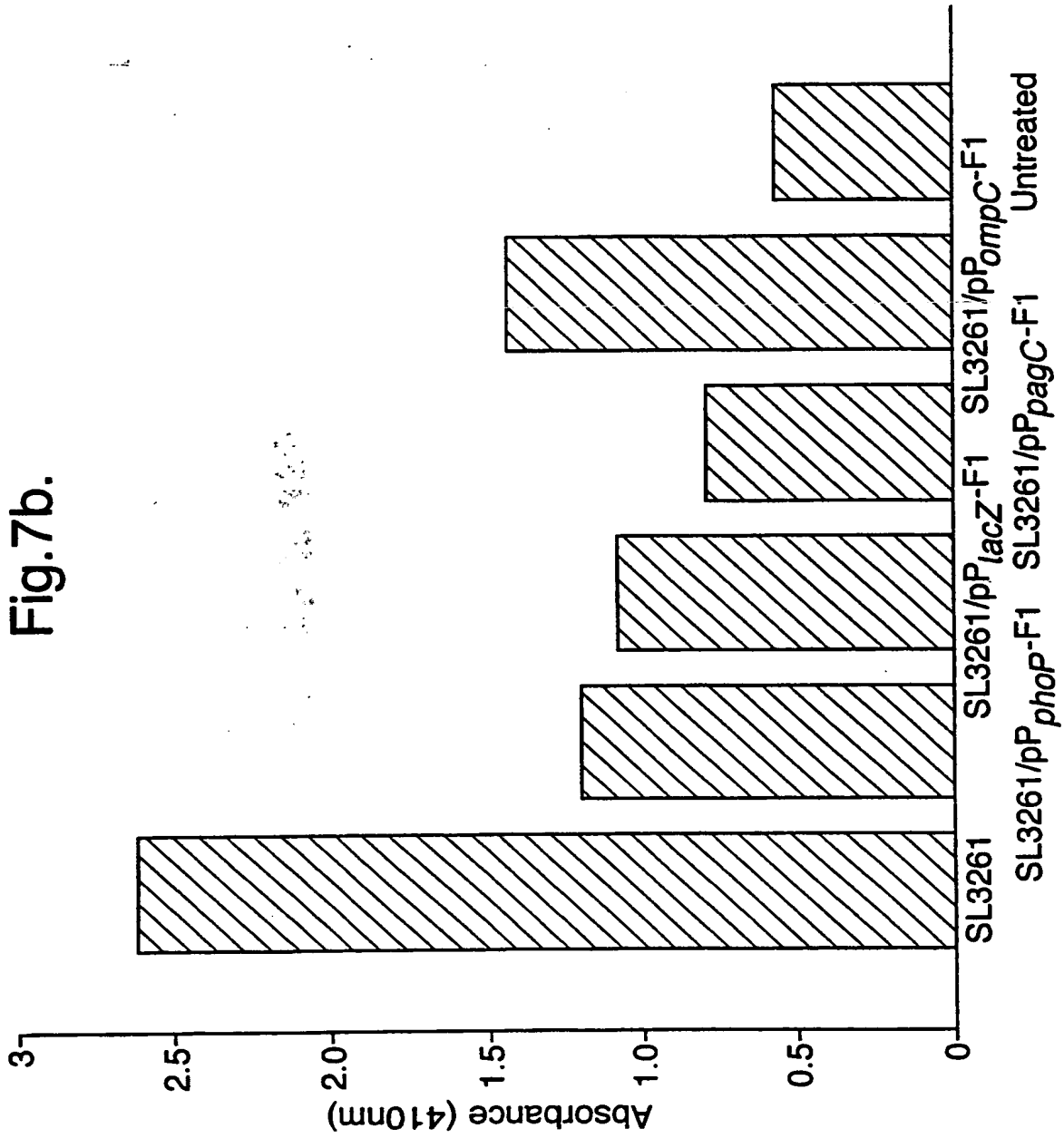

Absorbance (410nm)
0
0.5
1.0
1.5
2.0
2.5
3
SL3261
SL3261/pP$_{phoP}$-F1
SL3261/pP$_{lacZ}$-F1
SL3261/pP$_{pagC}$-F1
SL3261/pP$_{ompC}$-F1

Fig.7b.


Absorbance (410nm)
0
0.5
1.0
1.5
2.0
2.5
3
SL3261
SL3261/pP*phoP*-F1
SL3261/pP*lacZ*-F1
SL3261/pP*pagC*-F1
SL3261/pP*ompC*-F1
Untreated